A subset of GAF domains are evolutionarily conserved sodium sensors.

Martin Cann.

School of Biological and Biomedical Sciences, Durham University, South Road, Durham, DH1 3LE, United Kingdom.

E-mail: m.j.cann@durham.ac.uk

Phone: (+44) 191 334 1313

Fax: (+44) 191 334 1212

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Abstract.

Most organisms maintain a transmembrane sodium gradient for cell function. Despite the importance of Na^+ in physiology, no directly Na^+ responsive signalling molecules are known. The CyaB1 and CyaB2 adenylyl cyclases of the cyanobacterium *Anabaena* PCC 7120 are inhibited by Na^+. A D360A mutation in the GAF-B domain of CyaB1 ablated cAMP mediated autoregulation and Na^+ inhibition. Na^+ bound the isolated GAF domains of CyaB2. cAMP blocked Na^+ binding to GAF domains but Na^+ had no affect on cAMP binding. Na^+ altered GAF domain structure indicating a mechanism of inhibition independent of cAMP binding. Δ*cyaB1* and Δ*cyaB2* mutant strains did not grow below 0.6 mM Na^+ and Δ*cyaB1* cells possessed defects in Na^+/H^+ antiporter function. Replacement of the CyaB1 GAF domains with those of rat phosphodiesterase type 2 revealed that Na^+ inhibition has been conserved since the eukaryotic/bacterial divergence. CyaB1 and CyaB2 are the first identified directly Na^+ responsive signalling molecules that function in sodium homeostasis and we propose a subset of GAF domains underpins an evolutionarily conserved Na^+ signalling mechanism.

Introduction.

The ability to maintain appropriate intracellular inorganic ion concentrations when challenged by extracellular fluctuations is among the most ancient and fundamental cellular processes. Sodium is an essential cation whose intracellular levels can be maintained by primary and secondary transport, for example, by Na^+-ATPases and Na^+/K^+-ATPases in animal cells and H^+-ATPases, ion channels, and co-transporters in plant cells (Pedersen, 2005; Zhu, 2003). Typically, intracellular concentrations of Na^+ are maintained much lower than in the extracellular milieu. This transmembrane Na^+ gradient can be used as the basis for electrical signalling or can be coupled to electrochemically unfavourable solute flow.

Na^+ homeostatic response mechanisms enable cells to adapt to increases or decreases in environmental Na^+ and their study is of great interest in both agriculture and medicine. Salinity stress is one of many abiotic decertifying stressors that reduces agricultural output by 50% worldwide and more than 50% of global arable lands may be under salinity stress by 2050 (Boyer, 1982; Wang et al., 2003) Plants respond to salinity stress through a number of distinct mechanisms including the homeostatic transport of ions, control of water flux, and osmolyte biosynthesis (Hasegawa et al., 2000). Among the human population, many normotensive and hypertensive patients are “salt sensitive” and these patients show large fluctuations in blood pressure in response to salt repletion or depletion (Franco and Oparil, 2006). Despite these fundamental roles for Na^+ in biology, no molecular mechanism by which fluctuations in Na^+ concentration are directly detected and signalled has been identified. Identification of such a direct detection mechanism would be vital to understanding the biology of many medically and environmentally important Na^+-dependent processes.

The cyanobacteria are an excellent model for studying Na^+ stress response mechanisms. The identification of six specific histidine kinases and five response

regulators required for the full stimulation of salt-induced genes of *Synechocystis* PCC 6803 demonstrates that the bacterial two component system has a key role in Na^+ detection in this organism (Murata and Suzuki, 2006). The demonstration that NaCl dependent enhancement of heterocyst development in the filamentous N_2 fixing cyanobacterium *Anabaena* PCC 7120 was dependent upon cAMP production by the multi-domain CyaC adenylyl cyclase (AC) *in vivo* also raises the possibility that an individual protein domain may detect a Na^+ signal (Imashimizu *et al.*, 2005). A major component of CyaC is a dual GAF (found in cGMP phosphodiesterases, adenylyl cyclases, and FhlA [formate hydrogen lyase transcriptional activator]) domain motif. The ubiquitous GAF domain is an important site of signal perception in many eukaryotes and prokaryotes (Aravind and Ponting, 1997; Hurley, 2003). GAF domains from diverse species have equally diverse ligands including bilin chromophores in plants and cyanobacteria, haem in the DoS sensor of *Mycobacterium tuberculosis*, NO in the NorR sensor of *Escherichia coli*, 2-oxoglutarate in NifA of *Azotobacter vinelandii*, and the cyclic nucleotides cAMP and cGMP in cyanobacteria, unicellular parasitic eukaryotes, and mammals (D'Autreaux et al., 2005; Fischer et al., 2005; Gross-Langenhoff et al., 2006; Kanacher et al., 2002; Laxman et al., 2005; Little and Dixon, 2003; Rybalkin et al., 2003; Sardiwal et al., 2005).

The mammalian cyclic nucleotide phosphodiesterases (PDE) are integral to the regulation of cellular levels of cAMP and cGMP by controlling the rate of degradation (Martinez et al., 2002a; Zoraghi et al., 2004). At least eleven distinct families of PDE exist whose activity can be regulated by their N-termini (Francis et al., 2001; Houslay and Adams, 2003). Of these regulatory domains PDEs types 2, 5, 6, 10, and 11 possess GAF domains regulated by cyclic nucleotides (Erneux et al., 1981; Gross-Langenhoff et al., 2006; Mullershausen et al., 2003; Rybalkin et al., 2003; Zoraghi et al., 2005). The CyaB1 and CyaB2 ACs of *Anabaena* PCC 7120 also bind cAMP through one (CyaB1) or two

(CyaB2) N-terminal GAF domains to mediate positive feedback regulation of a carbon dioxide responsive AC domain (Bruder et al., 2005; Hammer et al., 2006; Kanacher et al., 2002; Martinez et al., 2005). Although cyclic nucleotide binding to GAF domains in multiple species is well characterized, molecules that control cyclic nucleotide regulation of GAF domain function are not known.

Here we demonstrate that Na^+ binds to and inhibits cAMP dependent GAF domain mediated positive feedback in the CyaB1 and CyaB2 ACs through blocking a conformational change associated with cAMP binding to the GAF domain. Genetic ablation of both the *cyaB1* and *cyaB2* genes gives strains defective in sodium homeostasis mediated by a defect in Na^+/H^+ antiporter function. Na^+ regulation of GAF domain function is conserved in the GAF domain of a mammalian PDE. CyaB1 and CyaB2 are therefore the first ever identified signalling molecules that respond directly to Na^+ and function in sodium homeostasis. Na^+ regulation of GAF domain function has also been evolutionarily conserved over a period of several billion years and may represent a widespread mechanism for Na^+ detection.

Results.

Na^+ inhibits the CyaB1 adenylyl cyclase of *Anabaena*.

The *cyaB1* (alr2266; http://www.kazusa.or.jp/cyano/Anabaena/) gene of *Anabaena* PCC 7120 codes for a protein consisting of two tandem GAF (GAF-A and GAF-B) domains, a PAS (found in periodic clock protein, aryl hydrocarbon receptor, and single minded protein) domain, and a C-terminal AC catalytic domain. Purified full length recombinant CyaB1 holoenzyme ($CyaB1_{1\text{-}859}$) is activated by inorganic carbon at pH 7.5 added as 10 mM $KHCO_3$ but not $NaHCO_3$ (Cann et al., 2003). This suggested that Na^+ blocked an aspect of intramolecular signalling and we therefore assessed the response of $CyaB1_{1\text{-}859}$ and a protein corresponding to only the CyaB1 AC catalytic domain ($CyaB1_{595\text{-}859}$) to a range of monovalent cations. Li^+ inhibited $CyaB1_{595\text{-}859}$ specific activity but there was no significant influence of Na^+ compared to basal activity (Figure 1A). In contrast $CyaB1_{1\text{-}859}$ specific activity was reduced two-fold in the presence of 20 mM Na^+ when compared to basal activity and other cations (Figure 1B). $CyaB1_{1\text{-}859}$ specific activity was not affected by Li^+ indicating that removal of N-terminal regulatory domains from CyaB1 revealed a small cation-binding pocket in the catalytic domain. We determined the dose response of $CyaB1_{1\text{-}859}$ inhibition by Na^+ with K^+ as control and confirmed that the reduction in specific activity occurred over a biologically relevant concentration range with an apparent $I.C._{50}$ of 14.3±2.2 mM (S.D.) for Na^+ (Figure 1C). As Na^+ is acting on recombinant protein the data is unambiguous and $CyaB1_{1\text{-}859}$ represents the first identified directly and specifically Na^+ regulated signalling molecule.

As the AC domain is not the site of action of Na^+ in the holoenzyme we investigated the role of the GAF domains in the Na^+ response. The GAF-B domain of $CyaB1_{1\text{-}859}$ is an autoregulatory domain that binds cAMP and up regulates AC activity by an unidentified mechanism while the GAF-A domain is of unknown function (Kanacher et al., 2002). We hypothesized that Na^+ inhibited AC activity by suppression of the cAMP-

mediated positive feedback loop and we reasoned that exogenous cAMP would compensate for this inhibition. In support of this reasoning Na^+ increased the $E.C._{.50}$ for cAMP activation of the AC domain to 0.4 μM from 0.17 μM in the presence of K^+ and Na^+ inhibition was not observed at 1 μM cAMP and above (95% C.I.) (Figure 2A). More specifically, Na^+ did not permit cAMP mediated activation of the AC domain at 30 nM exogenous cAMP and below (95% C.I.) indicating that Na^+ likely represents a mechanism to block uncontrolled activation of GAF domain positive feedback by low levels of cAMP. As $CyaB1_{1\text{-}859}$ autoregulation is mediated through the GAF-B domain we investigated Na^+ inhibition of recombinant proteins in which either the GAF-A ($CyaB1_{1\text{-}859}$D190A) or GAF-B ($CyaB1_{1\text{-}859}$D360A) domains were functionally compromised. The mutated aspartate forms part of a conserved $NKX_nFX_3\underline{D}E$ motif in mammalian PDEs essential for GAF domain functional integrity, assessed through cGMP binding, but does not bind cGMP (Martinez et al., 2002b; McAllister-Lucas et al., 1995). Na^+ down regulated $CyaB1_{1\text{-}859}$D190A specific activity ($E.C._{.50}$ for cAMP=1.48 μM) compared to K^+ ($E.C._{.50}$ for cAMP=0.67 μM). Similar to wild type enzyme Na^+ did not permit cAMP mediated activation of the AC domain at 30 nM and below and Na^+ inhibition was not observed at 1 μM cAMP and above (95% C.I.) (Figure 2B). At any given concentration of exogenous cAMP, fold inhibition of $CyaB1_{1\text{-}859}$D190A specific activity is reduced relative to wild type indicating that the GAF-A domain may have a subtle role in the inhibitory mechanism. $CyaB1_{1\text{-}859}$D360A specific activity was not responsive to Na^+ consistent with a role for Na^+ in the inhibition of cAMP mediated autoregulation (Figure 2C). As $CyaB1_{1\text{-}859}$D360A specific activity was maintained similar to the basal activity of the wild type enzyme (Figure 2A), the loss of inhibition is not an artefact of altered enzyme activity.

cAMP dependent Na^+ binding to the GAF domains of the CyaB2 adenylyl cyclase.

The *cyaB2* gene (all1904) of *Anabaena* PCC 7120 codes for a protein with an identical domain structure to CyaB1 (Katayama and Ohmori, 1997). In contrast to CyaB1, however, cAMP binds to and signals through both the GAF-A and GAF-B domains (Bruder et al., 2005; Martinez et al., 2005). The CyaB2 holoenzyme cannot be expressed as a recombinant protein but the tandem GAF domains of CyaB2 can be fused to the PAS and AC domains of CyaB1 to generate a chimeric recombinant protein (CyaB2-GAF-CyaB1) that enables study of the CyaB2 GAF domains (Bruder et al., 2005). Similar to CyaB1, the AC specific activity of the CyaB2 chimera is down regulated by Na^+ compared to other monovalent cations (Figure 3A). It is important to note that the specific inhibition of $CyaB1_{595\text{-}859}$ by Li^+ is not observed in $CyaB1_{1\text{-}859}$ or CyaB2-GAF-CyaB1 indicating that loss of the GAF/PAS domain region exposed an additional cation binding site but is most likely not of relevance *in vivo*. A dose response with Na^+ gives an apparent $I.C._{.50}$ of 24.5±0.9 mM (S.D.) (Figure 3B). The specific inhibitory effect of Na^+ is also compensated for by exogenous cAMP demonstrating that Na^+ has a similar activity in both CyaB1 and CyaB2 of inhibiting cAMP mediated autoregulation (Figure 3C).

To support a role for Na^+ binding to and modulation of GAF domain activity in CyaB1 and CyaB2 we sought evidence for a direct Na^+-GAF domain interaction. Unfortunately, the tandem GAF domain motif of CyaB1 cannot be expressed as a recombinant protein for these studies but the tandem GAF domain of the CyaB2 AC of *Anabaena* expresses at a high level (CyaB2 $GAFA/B_{58\text{-}445}$) (Martinez et al., 2005). As Na^+ inhibits the specific activity of the CyaB1 AC domain when expressed with either the CyaB1 or CyaB2 tandem GAF domain motif we reasoned that an analysis of CyaB2 $GAFA/B_{58\text{-}445}$ would inform our study of both the CyaB1 and CyaB2 enzymes.

SBFI is a fluorescent indicator specific for Na^+ and has a K_D for Na^+ (3.8 mM in the absence of K^+) of a similar order of magnitude as the $I.C._{.50}$ for CyaB2-GAF-CyaB1. We reasoned that adding a protein that specifically binds Na^+ to a SBFI/Na^+ mix would

manifest as a decrease in SBFI fluorescence emission. CyaB2 GAFA/B_{58-445} specifically bound Na^+ to a substantially greater degree than K^+ with a Hill Slope of 0.94±0.05 (S.D.) (Figure 4A). Na^+ therefore binds and functions specifically at the GAF domains and not an alternative region of the enzyme. A thermodynamic analysis gave a binding constant for Na^+ (K_i) of 302.3±21.1 μM Na^+ (S.D.), a value significantly lower than the $I.C._{50}$ values obtained by enzymology. Addition of cAMP to the quenching assay at an elevated concentration sufficient to saturate the necessarily high protein concentration in the assay blocked Na^+ binding to CyaB2 GAFA/B_{58-445} supporting the biochemistry and demonstrating that cAMP compensates for Na^+ inhibition by displacing Na^+ from the GAF domain (Figure 4B).

Given that Na^+ bound to a model tandem GAF domain protein and was displaced by cAMP we sought further evidence of the mechanism of Na^+ inhibition of GAF domain function. We used isothermal microcalorimetry to assess the effect of Na^+ on cAMP binding to CyaB2 GAFA/B_{58-445}. Microcalorimetry has been used successfully to demonstrate cAMP binding to the GAF-A domain of the TcrPDEB2 PDE of *Trypanosoma cruzi* and 2-oxoglutarate binding to the NifA GAF domain from *Azotobacter* (Diaz-Benjumea et al., 2006; Martinez-Argudo et al., 2004). Remarkably, Na^+ had no specific effect on the affinity of CyaB2 GAFA/B_{58-445} for cAMP with dissociation constants of 0.87±0.07 (basal conditions), 0.98±0.06 (50 mM Na^+), and 1.04±0.01 (50 mM K^+) μM cAMP (S.D.).

As cAMP is able to displace Na^+ but not vice versa we investigated the role of Na^+ in an event associated with the tandem GAF domain but which is independent of cAMP binding. Aromatic amino acids display chiroptical activity at near UV wavelengths. Chiroptical activity between 250 and 300 nm is particularly sensitive to changes in protein tertiary structure (Sreerama and Woody, 2000). Obtained circular dichroism spectra for CyaB2 GAFA/B_{58-445} with 50 mM K^+ or in the absence of salt are essentially identical both

in the presence and absence of cAMP (Figure 5). In contrast spectra in the presence of 50 mM Na^+ are significantly different. This demonstrated that Na^+ effected a change in the tertiary structure of CyaB2 GAFA/B_{58-445} and is the likely cause of the disruption in GAF domain activity.

CyaB1 and CyaB2 are required for sodium homeostasis at limiting sodium in *Anabaena*.

To determine whether the Na^+ inhibition of CyaB1$_{1-859}$ and CyaB2-GAF-CyaB1 *in vitro* was of functional significance *in vivo*, we examined the Na^+ homeostatic response of *Anabaena* PCC 7120 strains ablated for *cyaB1* or *cyaB2* gene function (Δ*cyaB1* and Δ*cyaB2* respectively) compared to wild type cells. The growth rates of wild type and Δ*cyaB1* cells in a standard defined medium for *Anabaena* culture containing normal (4 mM) Na^+ or elevated (40 mM) Na^+ were indistinguishable (data not shown). We therefore examined the homeostatic response of wild type, Δ*cyaB1*, and Δ*cyaB2* cells at limiting Na^+ concentrations. Significantly, Δ*cyaB1* and Δ*cyaB2* cells were unable to grow below 0.6 mM Na^+ while wild type cells were able to grow at concentrations at least as low as 0.2 mM Na^+ (Figure 6A). This effect is independent of osmolarity or ionic strength as identical results are obtained when the removed Na^+ is replaced with K^+. The nitrogen growth regime for Δ*cyaB1* cells did not affect the lethality at low Na^+ (data not shown). The *cyaA* gene (all1118) encodes a protein with an AC domain but no predicted GAF domains. The growth of Δ*cyaA* cells was indistinguishable from wild type consistent with a role for the GAF domain ensemble in Na^+ signalling rather than a non-specific effect of loss of an AC domain. N^6, 2'-O-dibutyryladenosine 3', 5'-cyclic monophosphate (db-cAMP) is a cell-permeable cAMP analogue that can be used to effect entry of cAMP into the cell. Inclusion of db-cAMP in the medium rescued growth of both Δ*cyaB1* and Δ*cyaB2*

cells at 0.2 mM Na^+ after extended culture periods (Figure 6B). This demonstrated that the inability to grow at limiting Na^+ in the strains defective in AC expression could be compensated for by cAMP.

Decreased environmental pH can compensate for a Na^+ deficiency in the growth of *Anabaena* (Abe et al., 1987). Consistent with this, growth of Δ*cyaB1* and Δ*cyaB2* cells was partially rescued in 0.2 mM Na^+ containing media at pH 7.0 while growth was not supported at pH 8.0 (Figure 6C). The ability of an increased H^+ concentration to compensate for lowered environmental Na^+ suggested that aspects of Na^+ dependent H^+ flux were abnormal in Δ*cyaB1* and Δ*cyaB2* cells grown at 0.2 mM Na^+. We therefore examined the phenotype of Δ*cyaB1* cells in more detail with respect to Na^+/H^+ antiporter activity. Of the pH sensitive fluorescent probes assessed for use in cyanobacteria, acridine orange has been demonstrated to be of general utility in monitoring intracellular ΔpH (Teuber et al., 2001). Partitioning of acridine orange into an acidic intracellular space has been used as a non-calibrated method to assess Na^+/H^+ antiporter activity in mammalian cells and cyanobacteria (Blumwald et al., 1984; Elanskaya et al., 2002; Reenstra et al., 1981; Wang et al., 2002). We therefore investigated Na^+-dependent intracellular and extracellular acidification in wild type and Δ*cyaB1* cells. Wild type cells pre-incubated for 24 hours at 0.2 mM Na^+ showed a partitioning of acridine orange into an acidic intracellular space when diluted into a Na^+-free assay buffer (Figure 7, phase I) and this partitioning was reversed on addition of 4 mM Na^+ due to an alkalization of the intracellular space (Figure 7, phase II). In contrast, Δ*cyaB1* cells showed an abnormal partitioning of acridine orange after pre-incubation at 0.2 mM Na^+ for 24 hours and this was not fully reversed by addition of 4 mM Na^+ (Figure 7) while Δ*cyaA* cells were indistinguishable from wild type (data not shown). Acridine orange partitioning was identical in wild type and Δ*cyaB1* cells grown at 4 mM Na^+ and in cells exposed to a

transient downshift from 4 to 0.2 mM Na^+ (data not shown). The requirement for prolonged exposure to 0.2 mM Na^+ to observe a phenotype in wild type versus Δ*cyaB1* cells suggested a requirement for new protein synthesis in the homeostatic response to low Na^+. Indeed, wild type cells incubated for 24 hours at 0.2 mM Na^+ in the presence of the translational inhibitor chloramphenicol (400 μg/ml) showed a defective phase I response compared to cells in the absence of inhibitor (data not shown). Δ*cyaB1* cells grown at 0.2 mM Na^+ therefore have a clear defect in Na^+-dependent intracellular acidification and alkalization. This indicated that Na^+/H^+ antiporter function is abnormal in Δ*cyaB1* cells grown under conditions of low Na^+.

Na^+ regulation is conserved in mammalian GAF domains.

As we have demonstrated that Na^+ specifically blocks cAMP mediated autoregulation of a model cyanobacterial tandem GAF domain with functional consequences for the Na^+ stress response *in vivo* we asked whether Na^+ regulation of GAF domain function is conserved among other species. Select cyclic nucleotide regulated GAF domains of the mammalian PDEs functionally couple to and regulate the activity of the CyaB1 AC domain in chimeric molecules (Bruder et al., 2006; Gross-Langenhoff et al., 2006; Kanacher et al., 2002). As we have defined the tandem GAF domain of the *Anabaena* ACs as the Na^+ responsive region of the protein we reasoned that introduction of mammalian PDE GAF domains to generate chimeric CyaB1 proteins would enable us to address specifically whether the mammalian GAF domains are able to respond to Na^+. A chimeric protein consisting of the rat PDE2 tandem GAF domain and CyaB1 PAS and AC domains (PDE2-GAF-CyaB1) gives a protein with cGMP activated GAF domains that in turn activate the CyaB1 AC domain (Kanacher et al., 2002). We examined the response of PDE2-GAF-CyaB1 to Na^+ with K^+ as control over a range of cGMP concentrations and found that Na^+ inhibited AC activity relative to K^+ under conditions of GAF domain

activation by cGMP (Figure 8A). Assay in the presence of a range of monovalent cations demonstrated that this effect was specific for Na^+ (Figure 8B). Inhibition was specific for Na^+ relative to K^+ over a broad concentration range and gives an $I.C._{.50}$ of 47.4±10.0 mM (S.D.) (Figure 8C). The principle of Na^+ regulation of GAF domain function is therefore conserved over a period of several billion years of evolution and can function in the context of structurally diverse proteins.

Discussion.

The CyaB1 and CyaB2 ACs of *Anabaena* represent the first identified signal transduction molecules whose activity is modulated specifically by Na^+ and are functionally required for sodium homeostasis. The dependence of Na^+ inhibition in CyaB1 on GAF-B domain functional integrity and ablation of the Na^+ response by exogenous cAMP demonstrated that Na^+ acts at the GAF domain to block cAMP mediated autoregulation at low cAMP concentrations. The necessity for this process *in vivo* is clear; without an inhibitor of autoregulation, activation of the AC domain would proceed unchecked and uncontrolled. By demonstrating conservation of Na^+ inhibition in a CyaB2-CyaB1 chimeric molecule we were able to use the well characterized isolated tandem GAF domain motif of CyaB2 to investigate the Na^+ binding mechanism. Na^+ but not K^+ bound effectively to the isolated GAF domains underpinning the results of the biochemistry. The observation that cAMP blocked Na^+ binding further supported the biochemical finding that exogenous cAMP ablated Na^+ inhibition of the holoenzyme.

Interestingly, Na^+ had no effect on the affinity of the GAF domains for cAMP demonstrating binding most likely occurred at distinct sites. A probable interpretation of the data is that Na^+ stabilized the GAF domain in a conformation that is unable to signal in the presence of low levels of cAMP. Increased cAMP displaced Na^+ and preferentially altered the structure of the GAF domain to a new conformation capable of signalling. Consistent with this hypothesis, non-cAMP bound Na^+-free GAF domains have a distinct tertiary structure compared to non-cAMP bound GAF domains in the presence of Na^+ as assessed by circular dichroism. Addition of cAMP to the Na^+ free GAF domains affects a distinct shift in tertiary structure that is consistent with signalling and most likely identical to that of the recent crystal structure (Martinez et al., 2005). The spectrum of the GAF domains in the presence of cAMP and Na^+ is harder to interpret, as the cAMP concentration used should mostly saturate the GAF domains. However, the fluorescence

quenching experiments demonstrate that a ten-fold molar excess of cAMP over protein does not displace all of the bound Na^+ (Figure 4B). The GAF domain-Na^+-cAMP spectrum may therefore represent an average of a minor contribution of the GAF-Na^+ bound state to the Na^+-free cAMP bound spectrum. Multiple conformational states have also been proposed for the tandem GAF domain motif of mammalian PDE5 (Rybalkin et al., 2003).

The $I.C._{50}$ for Na^+ for both CyaB1 and CyaB2 are seemingly high for an enzyme that must respond to much lower concentrations of Na^+ *in vivo*. Competition binding analysis with SBFI, however, gave an affinity of the CyaB2 GAF domains (measured as K_i) of 302.3±21.1 μM Na^+. We speculate that cAMP levels in the AC assays are sufficient to partially block Na^+ binding giving artificially raised values for the $I.C._{50}$ that are not reflective of the true affinity. The affinity of the GAF domains for Na^+ is well within the range of Na^+ concentrations likely to exist in the intracellular environment.

Importantly, the observed biochemistry is not an *in vitro* artefact. Both Δ*cyaB1* and Δ*cyaB2* cells show defects in Na^+ homeostasis at limiting Na^+ concentrations. A scenario consistent with the biochemistry is that local intracellular Na^+ concentrations at 4 mM extracellular Na^+ are sufficient to block cAMP mediated autoregulation of CyaB1 (Figure 9A). A drop in intracellular Na^+ precipitated by a fall in extracellular Na^+ then permits autoregulatory activation of the AC by basal cAMP concentrations (Figure 9B). The mechanism of cell death at limiting Na^+ is most likely due to defects in Na^+/H^+ antiporter function. Na^+/H^+ antiporters essential for survival at low environmental Na^+ have been characterized in the model cyanobacterium *Synechocystis* PCC 6803 (Mikkat *et al.*, 2000; Wang *et al.*, 2002). Mutations in the sll0273 (*nhaS2*) and slr1595 (*nhaS4*) genes encoding Na^+/H^+ antiporters have distinct defects in acridine orange partitioning. The observation that Δ*cyaB1* cells had a similar defect in an identical phenotypic assay argues persuasively that CyaB1 is required to regulate the activity of Na^+/H^+ antiporters required for growth at

low Na^+ (Figure 9B). Unfortunately we were unable to detect an increase in cellular cAMP in response to a drop in medium Na^+. This finding is not entirely surprising as the contribution of CyaB1 and CyaB2 to the cellular cAMP pool is not detectable (Katayama and Ohmori, 1997). We were, however, able to rescue both mutant strains with exogenous cAMP demonstrating that the Na^+ homoeostasis defects were caused by an inability to produce cAMP in response to a drop in extracellular Na^+.

The mammalian PDEs catalyze the hydrolysis of cyclic nucleotides and are an important mechanism for regulating cyclic nucleotide levels in the cell (Baillie et al., 2005). The GAF domains of the mammalian PDEs bind cAMP or cGMP and regulate the activity of the PDE catalytic domain (Martinez et al., 2002a; Zoraghi et al., 2004). Replacement of the tandem GAF domain motif of CyaB1 with those of mammalian PDEs permits activation of the AC domain by the specific cyclic nucleotide that binds the PDE GAF domain motif. Such chimeric molecules are excellent tools to ask whether Na^+ regulates mammalian GAF domains by isolating them from the remainder of the PDE molecule. Na^+ inhibited the function of the cGMP binding GAF domains of rat PDE type 2. Consistent with a role for Na^+ in blocking GAF domain activation, Na^+ had no effect on AC activity in the absence of exogenous cGMP but inhibited AC specific activity in the presence of cGMP. The difference in the requirements for cyclic nucleotide concentrations for Na^+ inhibition between $CyaB1_{1\text{-}859}$/CyaB2-GAF-CyaB1 (low concentrations) and PDE2-GAF-CyaB1 (elevated concentrations) indicates that the exact mechanism of Na^+ regulation may differ between enzymes. This may, however, be a reflection of the differing topology of GAF domain structure between enzymes (Martinez et al., 2005) and does not preclude the possibility of a similar site of action. Inhibitory Na^+ concentrations for the PDE2 chimera are consistent with intracellular Na^+ concentrations of mammalian cells exposed to high extracellular Na^+ (for example see (Efendiev et al., 2003; Komlosi et

al., 2003) indicating that regulation of cyclic nucleotide levels by PDEs can serve as a mechanism to directly respond to changes in Na^+ concentration.

The demonstration of functional regulation of GAF domain activity in *Anabaena* by Na^+ and the conservation of this biochemistry in mammalian PDEs demonstrates that a subset of GAF domains represent a mechanism for Na^+ detection and signalling conserved over two billion years of evolution.

Experimental Procedures.

Recombinant proteins: The $CyaB1_{595-859}$, $CyaB1_{1-859}$ (wild type, D190A, and D360 mutations), CyaB2-GAF-CyaB1, PDE2-GAF-CyaB1, PDE10A1-GAF-CyaB1, and CyaB2 $GAFA/B_{58-445}$ were expressed and purified as previously described except that NaCl was omitted from all dialysis buffers (Bruder et al., 2005; Kanacher et al., 2002; Martinez et al., 2005).

Adenylyl cyclase assays: The AC activity of all wild type and mutant AC proteins was assessed in a final volume of 100 μL (Salomon et al., 1974). Reactions contained 22% glycerol, 50 mM Tris.HCl pH 7.5 as buffer, 10 mM $MgCl_2$ as divalent metal cofactor, and 75 μM [α-^{32}P]ATP (25 kBq) as substrate unless otherwise indicated. 2 mM [2, 8-^{3}H]cAMP was added to the terminated assays to determine yield during product isolation. All assays were performed at 37^{o}C. Assay conditions were adjusted to keep substrate conversion <10% unless otherwise indicated. The data represent the means of independent experiments and error bars represent the standard error. Absent error bars indicates that the S.E.M. was smaller than the symbol used to indicate the data point.

Sodium binding fluorescence-quenching assay: Sodium fluorescence was measured using a Jasco FP-6200 Spectrofluorimeter. Assays were performed in a final volume of 50 μL and contained 25% glycerol, 50 mM Tris.HCl pH 7.5 as buffer, 10 mM $MgCl_2$, and, where required, 0.5 μM sodium-binding benzofuran isophthalate or potassium-binding benzofuran isophthalate (SBFI/PBFI; Molecular Probes) and 3.8 mM NaCl or KCl. Fluorescence was excited at 340 nm and 380 nm and excitation measured at 505 nm using a band width of 5 nm for both excitation and emission wavelengths. Emission in the absence of indicator was subtracted from all data points. Error bars represent the S.E.M.

376

377 *Isothermal microcalorimetry*: Binding of cAMP to CyaB2 GAFA/B_{58-445} was assessed by
378 titration isothermal calorimetry using a MicroCal VP-ITC and data analyzed using
379 dedicated Microcal/Origin® software. A 1.42 ml volume containing 50 mM Tris.HCl pH
380 7.5, 25% glycerol, 2 mM $MgCl_2$, and 30 μM CyaB2 GAFA/B_{58-445} in the presence or
381 absence of 50 mM salt was titrated with cAMP in the same buffer. The heat of dilution of
382 cAMP into buffer alone was subtracted from all data.

383

384 *Circular dichroism*: Changes in the tertiary structure of CyaB2 GAFA/B_{58-445} were
385 monitored using a Jasco J-810 Spectropolarimeter. Briefly, a 500 μL volume containing
386 50 mM Tris.HCl pH 7.5, 25% glycerol, 2 mM $MgCl_2$, and 27 μM CyaB2 GAFA/B_{58-445}
387 with or without salt and cAMP was scanned at 50 nm sec^{-1} with a band pass of 2 nm and
388 response time of 4 sec. Each spectrum was acquired 8 times and corrected for activity in
389 the absence of protein. The data is representative of several individual experiments.

390

391 *Cyanobacterial strains and growth*: Wild type and mutant strains of *Anabaena* PCC 7120
392 were grown in standard BG11 medium supplemented with 20 mM TES-NaOH pH 8.0
393 without combined nitrogen at 30^{o}C under a photosynthetically active light regime of ≈ 30
394 μmols m^{-2} s^{-1} (Katayama and Ohmori, 1997). Media containing defined amounts of
395 sodium were prepared by the addition of NaCl to BG11 and all other salts were added
396 with potassium as cation. Adjustments to medium pH were performed using 20 mM TES-
397 KOH (pH 7.0 or 8.0). For phenotypic assays, cells were grown to the mid log phase and
398 media replaced with BG11 20 mM TES-KOH pH 8.0 containing 0.2 mM NaCl for 24
399 hours. Growth experiments were transferred to 12-well plates for photography.
400 Chlorophyll measurements were performed as previously described (Arnon et al., 1974).

401

Measurement of intracellular acidification: Intracellular acidification was assessed using the acridine orange fluorescence quenching technique (Blumwald et al., 1984). A 25 μg protein equivalent in 6 μl of harvested cells pre-incubated at 0.2 mM Na^+ was diluted into 2 ml of Na^+ free assay buffer (0.8 M mannitol, 10 mM HEPES pH 7.5, 1 μM acridine orange). 4 mM Na_2SO_4 was added to the assay where indicated. Fluorescence spectroscopy was carried out using a Jobin-Yvon Fluorolog FL3-22 spectrofluorimeter. Assays were performed in a 1 cm x 1 cm cross-section cuvette equipped with a magnetic stirrer and held at 30^{o}C. The samples were irradiated at 492 nm and the intensity at 530 nm was recorded using a time driven acquisition mode. Both monochromators were set to a band pass of 2.5 nm. The observed intensity/time profiles were corrected for fluctuations in the excitation source and for quenching of acridine orange in a mock assay. Results shown are representative of several independent experiments.

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563 **Figure Legends.**

564 **Figure 1.** (A) Response of the CyaB1 catalytic domain to monovalent cations. 0.6 μM
565 $CyaB1_{595-859}$ was assayed with 1 mM Mg^{2+}-ATP and 20 mM salt (n=8). (B) Response of
566 the CyaB1 holoenzyme to monovalent cations. 25 nM $CyaB1_{1-859}$ was assayed with 75 μM
567 Mg^{2+}-ATP and 20 mM salt (n=8). (C) Dose response of the CyaB1 holoenzyme with salt.
568 25 nM $CyaB1_{1-859}$ was assayed with 75 μM Mg^{2+}-ATP at increasing NaCl (squares) or KCl
569 (triangles) concentrations (n=6). All differences except those of the basal activities were
570 significant (95% C.I.)

571

572 **Figure 2.** (A) Dose response of the CyaB1 holoenzyme with cAMP. 10 nM $CyaB1_{1-859}$
573 was assayed with 75 μM Mg^{2+}-ATP at increasing cAMP. All substrate conversion rates
574 are <12% (n=6). (B) Dose response of the CyaB1 GAF-A mutant with cAMP. 100 nM
575 $CyaB1_{1-859}$D190A was assayed with 75 μM Mg^{2+}-ATP at increasing cAMP (n=4). (C)
576 Dose response of the CyaB1 GAF-B mutant with cAMP. 100 nM $CyaB1_{1-859}$D360A was
577 assayed with 75 μM Mg^{2+}-ATP at increasing cAMP (n=6). All dose responses are
578 performed in the presence of 50 mM NaCl (squares) or KCl (triangles).

579

580 **Figure 3.** (A) Response of the CyaB2-GAF-CyaB1 chimera to monovalent cations. 480
581 nM CyaB2-GAF-CyaB1 was assayed with 75 μM Mg^{2+}-ATP, 0.7 μM cAMP, and 50 mM
582 salt (n=6). (B) Dose response of the CyaB2-GAF-CyaB1 chimera with salt. 480 nM
583 CyaB2-GAF-CyaB1 was assayed with 75 μM Mg^{2+}-ATP and 0.7 μM cAMP at increasing
584 NaCl (squares) or KCl (triangles) concentrations (n=6). (C) CyaB2-GAF-CyaB1 chimera
585 assayed with 75 μM Mg^{2+}-ATP, 50 mM salt, and cAMP as indicated (n=4).

586

Figure 4. (A) Cation binding to CyaB2 GAF domains. Fluorescence quenching of SBFI (squares) or PBFI (triangles) was plotted against CyaB2 GAFA/B_{58-445} concentration in the presence of NaCl or KCl respectively (n=3). Inset; derived Hill Plot of the data for SBFI. (B) cAMP blocks Na^+ binding to GAF domains. Fluorescence quenching of SBFI with 500 μM CyaB2 GAFA/B_{58-445} and 5 mM cAMP (n=4).

Figure 5. Circular dichroism spectropolarimetry of the CyaB2 tandem GAF domain. Analysis of CyaB2 GAFA/B_{58-445} without cAMP (dashed lines) or with 300 μM cAMP (solid lines) with no salt (green), 50 mM NaCl (blue), or 50 mM KCl (red).

Figure 6. (A) The response of wild type, Δ*cyaA*, Δ*cyaB1*, and Δ*cyaB2* cells to limiting Na^+ *in vivo*. (B) Rescue of the limiting Na^+ growth defect of Δ*cyaB1* and Δ*cyaB2* cells by exogenous cAMP. (C) Rescue of the limiting Na^+ growth defect of Δ*cyaB1* and Δ*cyaB2* cells by an increase in H^+ concentration. 7 and 8 denote growth pH.

Figure 7. Intracellular acidification assessed by acridine orange quenching in wild type and Δ*cyaB1* cells at limiting Na^+. Change in acridine orange fluorescence intensity of cells was plotted as a function of time. Cells and Na^+ were added to the assay at the indicated time point. I, H^+ uptake. II, H^+ efflux.

Figure 8. (A) Dose response of the Rat PDE2 CyaB1 chimera with cGMP. 0.44 μM PDE2-GAF-CyaB1 was assayed with 75 μM Mg^{2+}-ATP at increasing cGMP in the presence of 50 mM NaCl (squares) or KCl (triangles) (n=8). (B) Response of the Rat PDE2 GAF domains to monovalent cations. 0.44 μM PDE2-GAF-CyaB1 was assayed with 75 μM Mg^{2+}-ATP and 50 mM salt (n=4). (C) Dose response of the Rat PDE2 CyaB1

612 chimera with salt. 0.44 μM PDE2-GAF-CyaB1 was assayed with 75 μM Mg^{2+}-ATP with
613 increasing NaCl (squares) or KCl (triangles) (n=8).
614
615 **Figure 9.** Model for CyaB1 function *in vivo*. (A) Local concentrations of Na^+ at CyaB1 *in*
616 *vivo* are sufficient to block autoregulatory activation of CyaB1. (B) Reduced local
617 concentrations of Na^+ on lowering of extracellular Na^+ permit autoregulatory activation of
618 CyaB1 and regulation of Na^+/H^+ antiporter function. The dotted line between CyaB1 and
619 the Na^+/H^+ is not proven to be direct.
620

Figure 1A.

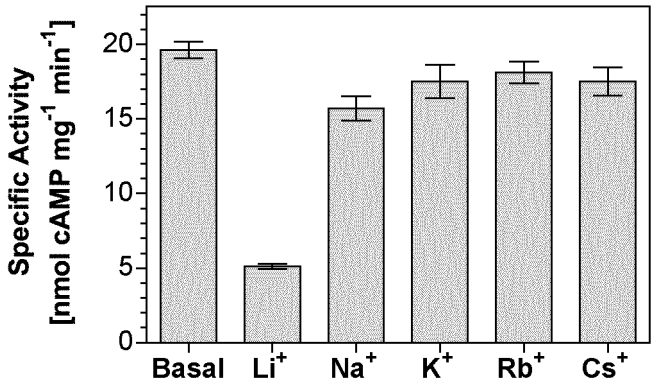
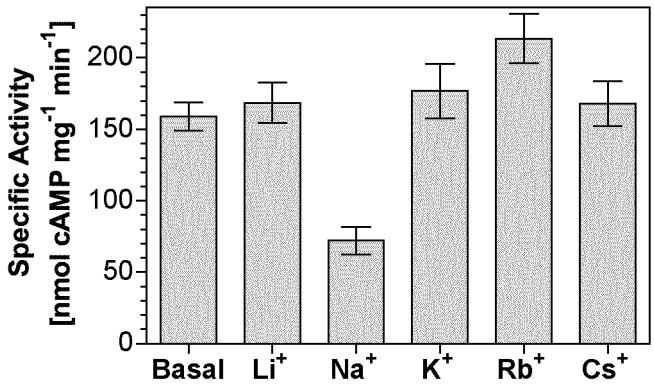


Figure 1B.



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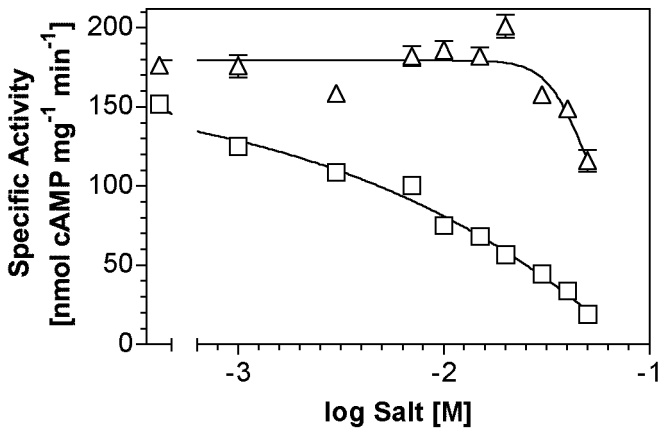

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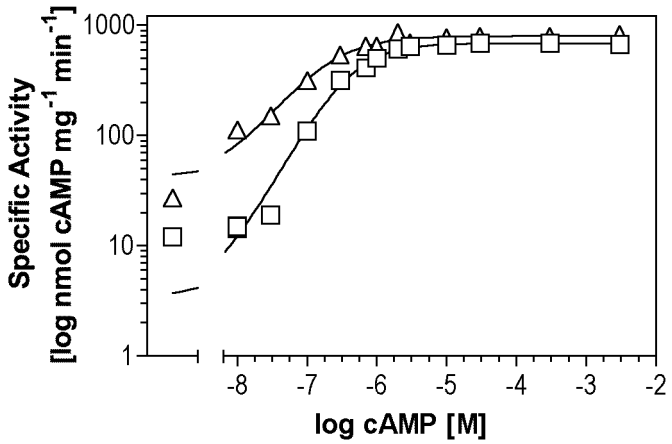

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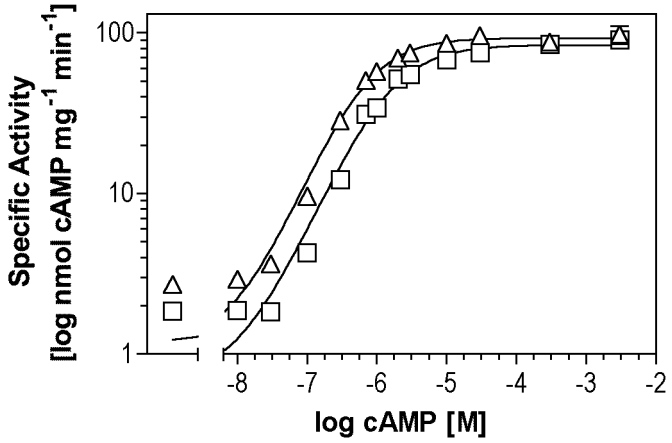

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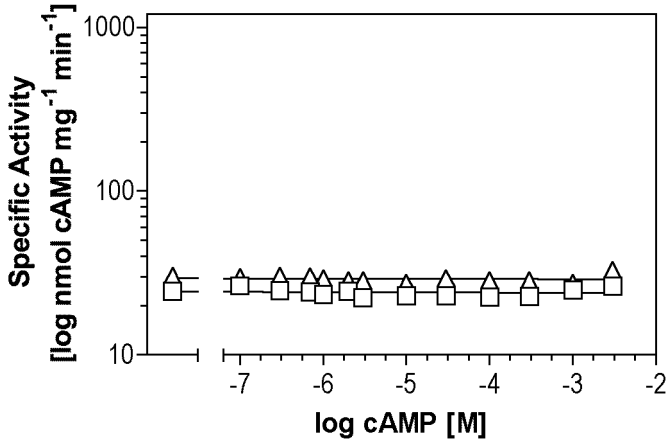
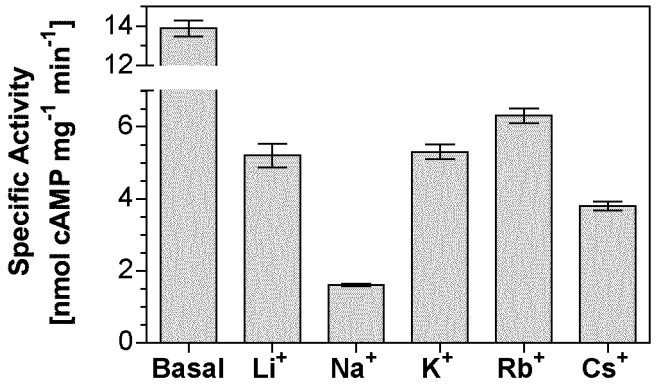


Figure 3A.



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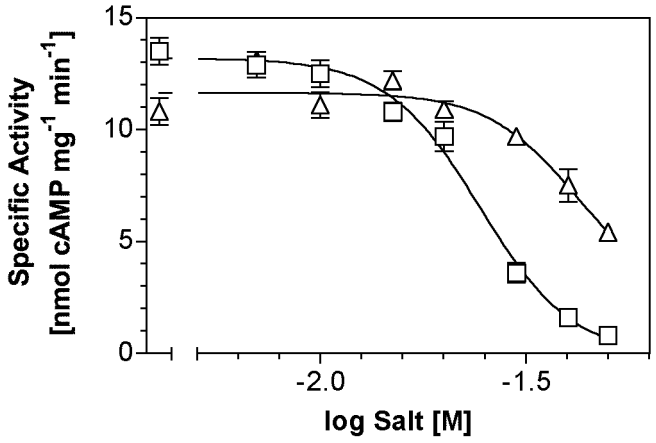

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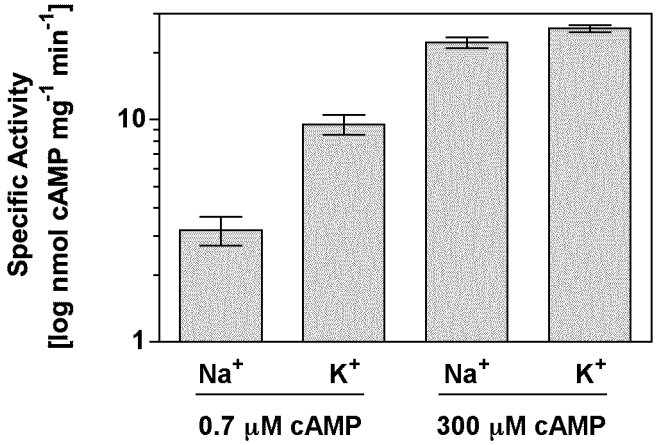
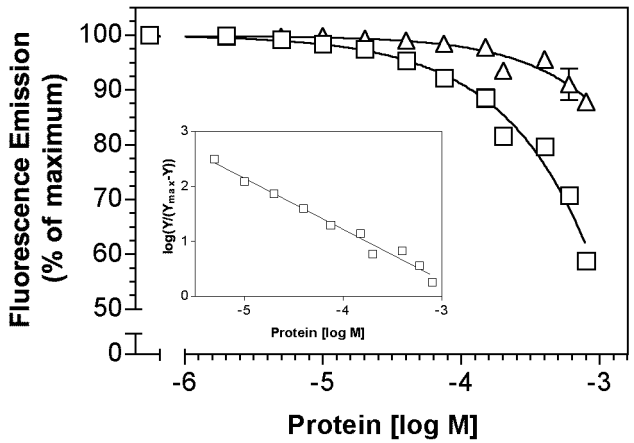


Figure 4A.



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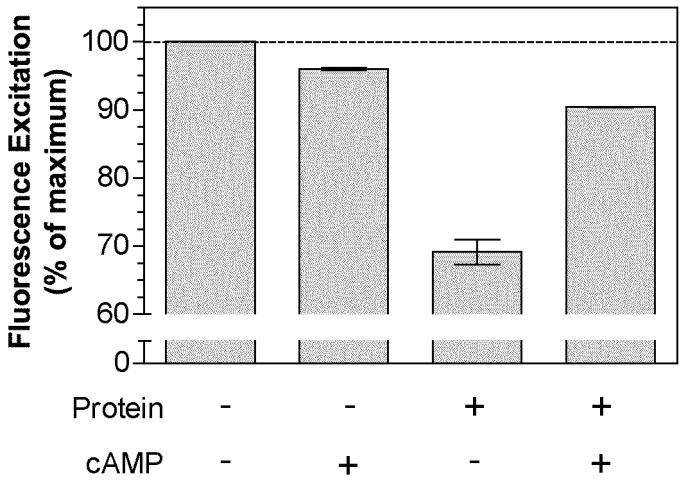
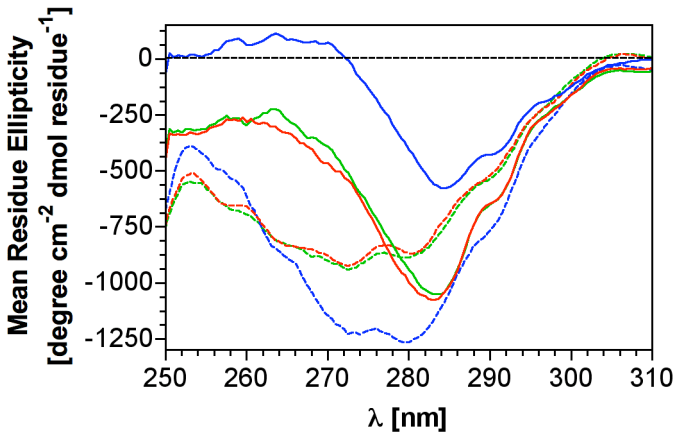


Figure 5.


Mean Residue Ellipticity
[degree cm-2 dmol residue-1]
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-750
-1000
-1250
250
260
270
280
290
300
310
λ [nm]

Figure 6A.

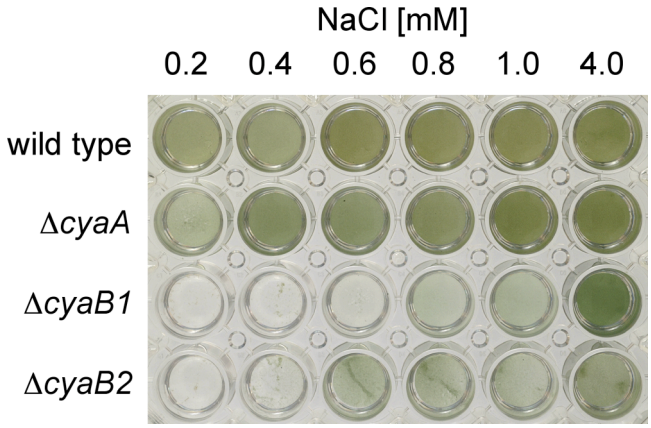


Figure 6B.

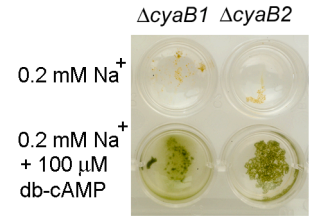
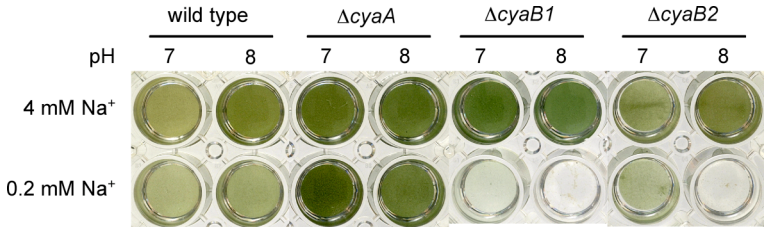


Figure 6C.



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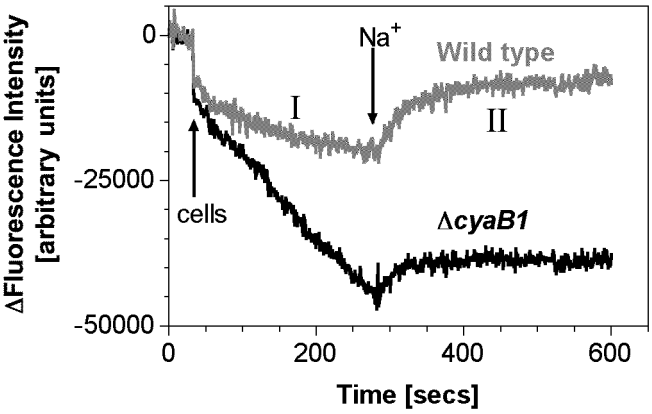
ΔFluorescence Intensity [arbitrary units]
0
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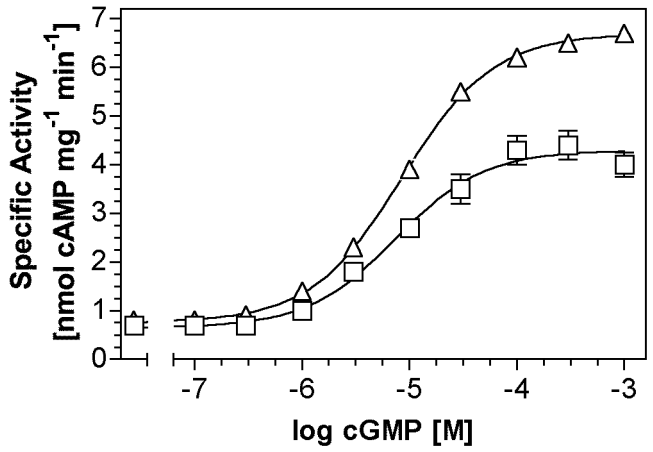
-50000
0
200
400
600
Time [secs]
Na+
Wild type
I
II
cells
ΔcyaB1

Figure 8A.



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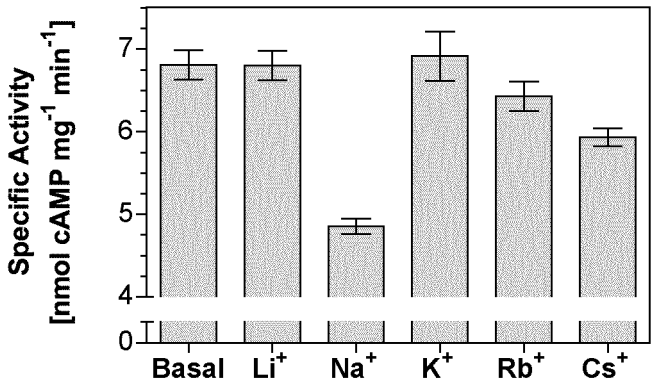


Figure 8C.

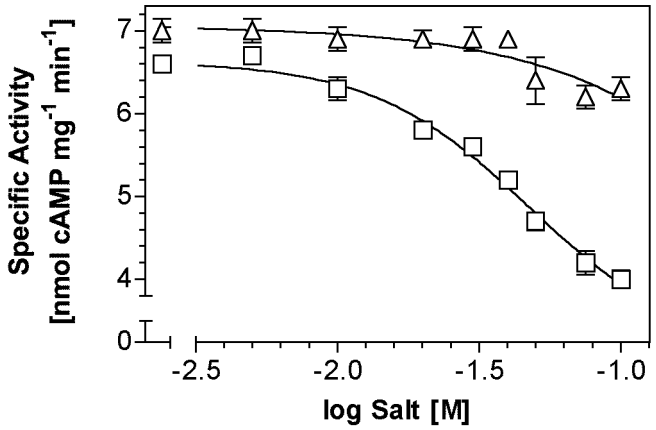
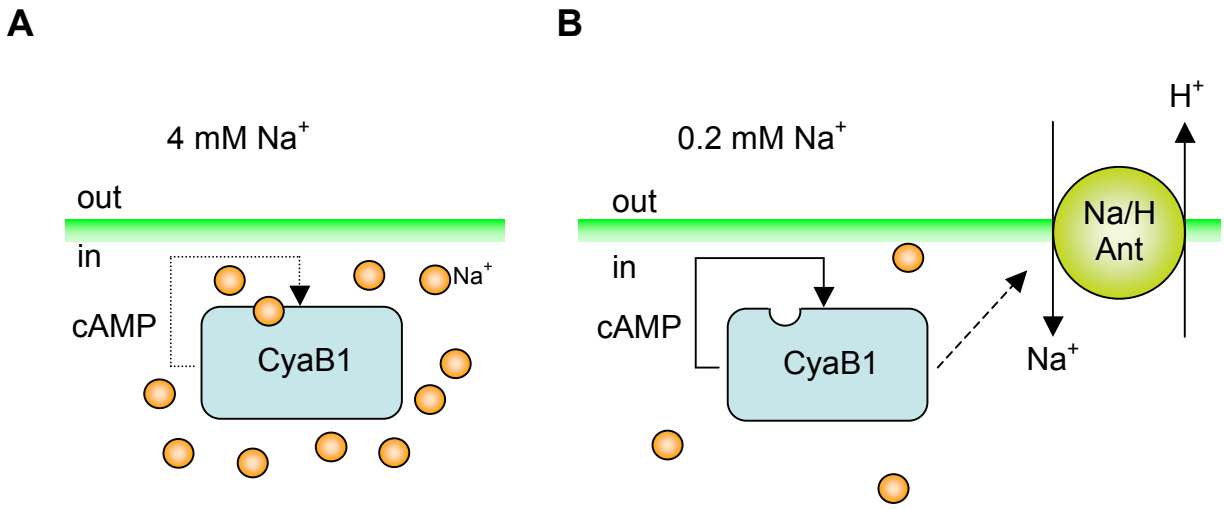


Figure 9.


A
4 mM Na+
out
in
cAMP
CyaB1
Na+
B
0.2 mM Na+
H+
out
in
cAMP
CyaB1
Na/H
Ant
Na+